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1. Conner et al. (1983) PNAS 80: 278-282.
2. Rollini et al. PNAS (1985 Nov) 82(12): 7197-7201.
3. Gorski et al. IMMUNOGENTICS (1987) 25(6):379-402.
4. de Preval et al. IMMUNOGEENTICS (1987) 26(4-5): 249-257
5. Irle et al. J. EXPERIMENTAL MEDICINE (1988 Mar 1) 167(3): 853-872.
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DNA typing of *HLA-DR*$_\beta$ chain genes can discriminate between undetected alleles and real homozygotes

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Abstract. The polymorphism of HLA-DR antigens has been studied by Southern blot hybridization under conditions specific for the detection of the *DR*$_\beta$ chain genes. Haplotype-specific patterns were defined with DNA from DR1, 2, 3, 4, 7, w8, w11, w12, and W13 homozygous typing cells, with restriction enzymes Eco RI, Bgl I, and Pvu II. Certain serological specificities, such as DR2, DR3, and DR7, can be encoded by distinct allelic forms of *DR*$_\beta$ chain genes. The procedure of "DNA typing" was applied to family analysis of individuals expressing only a single DR specificity upon serological typing. Three cases are described here: (1) in family GR, phenotypic DR 7 homozygotes correspond to genomic heterozygotes, and a novel *DR7* allele is described: (2) in family RU, the genes corresponding to a serologically undetected (blank) *DR* allele were identified by restriction fragment length polymorphism (RFLP); this novel *DR* haplotype has an RFLP pattern similar to those of the DRw52 family, even though this specificity was not expressed on the DR-blank lymphocytes; (3) in family RG, there is no blank allele, but a homozygote RFLP situation at the *DR* subregion.

Introduction

The *HLA-D* region of the human major histocompatibility complex (MHC) codes for class II or Ia antigens. These class II antigens are the products of MHC-linked immune response genes which control the ability to trigger an immune response against foreign antigens (Nagy et al. 1981). The Ia molecules consist of two transmembrane glycoproteins, the α (M_r 33000–35000) and the β (M_r 27000–29000) chains. In man three biochemically distinct groups of polymorphic class II antigens have been described, HLA-DR, -DQ, and -DP, encoded in three distinct subregions within the MHC (Kaufman et al. 1984, Mach et al. 1986). An important characteristic of Ia molecules is the extensive allelic polymorphism which is responsible for the self-restriction of cell-cell interaction within immunocompetent cells of each individual (Schwartz 1985). This polymorphism is of importance for organ transplantation since matching of class II loci, and of HLA-DR in particular, is essential for graft survival (Opelz 1984). Another relevant aspect of this polymorphism is that certain haplotypes are linked to susceptibility to certain diseases (reviewed by Möller 1983).

The established methodology for the analysis of HLA class II polymorphism involves serological, immunochemical, and cellular reagents (see reviews by Albert et al. 1984). All these procedures rely on the detection of the various epitopes of class II antigens and characterize the polymorphism and the complexity of the MHC system at the phenotypic level. The methodology of gene cloning and sequencing has led to a more direct analysis of the polymorphism and complexity of class II genes at the genotypic level, based on hybridization of cloned HLA class II genes to DNA restriction fragments (Wake et al. 1982). This procedure relies on polymorphic differences at certain restriction-enzyme sites and on allelic differences in the length of DNA fragments specific for individual class II genes (RFLP). This technique of "DNA typing" was first used to detect polymorphism of *DR* and *DQ* β chain genes (Wake et al. 1982).

Each of the three subregions, *DR, DQ, DP*, consists of a number of loci encoding α and β chains (Spielman et al. 1984, Auffray and Strominger 1986, Mach et al. 1986). In the case of HLA-DR (Fig. 1), there is a single, nonpolymorphic, α chain gene and several polymorphic β chain genes (Long et al. 1983). These have been linked on a molecular map (Spies et al. 1985, Rollini et al. 1985), and they show more than 90% sequence homology.

The present study is focused on *HLA-DR*$_\beta$ chain

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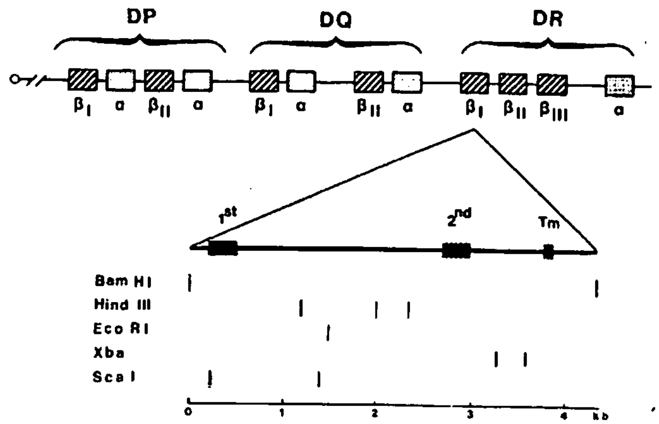


Fig. 1. Organization of *HLA-D* region and molecular map of the DR_β gene fragment used as hybridization probe. The *top line* represents a map of the *HLA-D* region. The number of DR_β chain genes is derived from a study by Rollini and co-workers (1985) and may vary in different haplotypes (Böhme et al. 1985). The *middle line* represents the human genomic fragment subcloned into pBR 322 and used as DR_β probe in Southern experiments (Fig. 2). This human gene fragment contains the exons (*filled boxes*) coding for the first and the second extracellular domains and for the transmembrane region of the DR_β polypeptide chain. A map with five enzymes is presented; the scale is in kilobases (kb)

genes and the experimental conditions were chosen to detect *DR* and not *DQ* or *DP* β chain genes. DNA patterns of homozygous typing cells (HTCs) derived from genetically homozygous donors (consanguineous marriages) were used to interpret RFLP patterns of heterozygous individuals in family studies. An interesting correlation was observed between certain restriction DNA patterns and the known serological supertypic specificities (DRw52/DRw53). This analysis was focused on the interpretation of family pedigrees in cases where one or several individuals express only a single DR serological specificity. Three observations were made: some individuals who are phenotypically homozygous were found to be genetically heterozygous with a "split" of the serological specificity at the DNA level; some individuals with a "blank" haplotype by serology have indeed a serologically unrecognized *HLA-DR* allele with a novel characteristic RFLP pattern corresponding to the "blank" haplotype; finally, some phenotypically homozygous individuals are homozygous by DNA genotyping. These "homozygous" HLA-DR phenotypes can now be interpreted in light of the RFLP pattern and new undetected alleles can be identified at the DNA level.

Materials and methods

Source of DNA. The origin and immunological typing of cell lines and blood samples used are described in Table 1.

Table 1. HLA phenotype of cells used in hybridizations

Donors		Consang.	HLA							Origin	Source
Form	Name		A	B	C	D	DR	DQ	DP		
HTC	MLV	+	w32	27	w2	w1	1	w1	w2	Leiden	Giphart
HTC	PHS	+	3	7	w7	w2	2	w1	w4	Leiden	Giphart
HTC	AVL	+	1	8	w7	w3	3, w52	w2	w4	Leiden	Giphart
HTC	QBL	+	26	18	w5	w3	3, w52	w2	ws2	Leiden	Giphart
HTC	HAR	+	1	8	w7	w3	3, w52	w2	w4	Leiden	Giphart
HTC	CAA	+	1	8	na	w3	3, w52	w2	w2	Leiden	Giphart
HTC	BSM	+	2	w62	w3	w4	4, w53	w3	w2	Leiden	Giphart
HTC	ATH	+	25	18	na	w11	5, w52	w3	w2	Leiden	Giphart
HTC	HHK	+	3	7	w7	w18	w13, w52	w1	w4	Leiden	Giphart
HTC	MANN	+	29	44	na	w7	7, w53	w2	w2	Copenhagen	Svegaard
HTC	BM9	+	2	w35	w4	w8	w8	na	na	Bergamo	Ferrara
HTC	BM16	+	2	18	w7	DB6	w12, w52	na	na	Bergamo	Ferrara
LCL	H9	–	1, 2	7, 8	na	w2, 3	2, 3	w1, 2	w1, 2	Bethesda	Shaw
LCL	M14	–	1, 2	7, 8	na	w2, 3	2, 4	w1, 2	w1, 2	Bethesda	Shaw
LCL	PM1	–	1, 2	7, 8	na	w2, 3	2, 3	w1, 2	w1, 2	Bethesda	Shaw
LCL	R5	–	1, 2	7, 8	na	w2, 3	2, 3	w1, 2	w1, 2	Bethesda	Shaw
F	RG	see legend to Fig. 7								Bergamo	Ferrara
F	RU	see legend to Fig. 6								Bergamo	Ferrara
F	GR	see legend to Fig. 5								Bergamo	Ferrara

HTC, homozygous typing cell; LCL, lymphoblastoid cell line (EBV) (transformed); F, donors from a family; Consang., donor issued from consanguineous marriage (first cousins); na, not available

Human genomic DNA. DNA preparations were adapted from the method of Kunkel and co-workers (1977).

DNA digestions. Routinely 10 μg of genomic DNA was digested to completion with either Eco RI, Bgl I, Pvu II, or Pst I (6 units/μg^{-1}). Phage lambda DNA (0.6 μg/20 μl) were digested with 2 units/μg^{-1} at 37 °C with either Eco RI or Hind III. The phages βDR 22, βDQ 18, and βDP 11 are genomic clones containing entire β chain genes.

Probe used for Southern hybridization. Plasmid pP14 BamG was constructed by subcloning into pBR322 a 4.3 kb Bam HI genomic fragment derived from a $DRw6_{\beta}I$ gene cloned into phage Charon 30 (Gorski et al. 1985). A restriction map of the Bam HI insert is presented in Figure 1. This human DNA fragment contains almost all the coding sequence of the $DR_{\beta}I$ gene, including exons coding for the first and the second extracellular domains and for the transmembrane segment.

Electrophoresis and transfer of DNA fragments. DNA fragments were depurinated, denatured, neutralized, and transferred according to the method of Southern (1975). Nitrocellulose filters (0.22 μ, Schleicher and Schuell, Dassel, Federal Republic of Germany) and GeneScreen Plus membrane (New England Nuclear, Boston, Massachusetts) were used in transfer experiments.

Prehybridization, hybridization, and washes. After transfer, the filters were rinsed with 4 × SSC (1 × SSC = 150 m*M* NaCl, 15 m*M* trisodium citrate). The DNA was fixed to nitrocellulose at 80 °C for 2 h, and to GeneScreen Plus by exposure to UV light (325 nm) for 2 min. Filters were presoaked in 5 × SSC, 5 × Denhardt's solution for 1 h at 68 °C with gentle shaking, and then prehybridized for 4-6 h at 68 °C in 1 × Denhardt's solution, 0.75 *M* NaCl, 5 m*M* ethylenediaminetetraacetate, 50 m*M* sodium phosphate buffer, pH 7.0, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 250 μg/ml^{-1} denatured herring sperm DNA. Hybridization was carried out for 15 h at 68 °C in the same buffer as used for prehybridization with 1 × 10^{6} cpm/ml^{-1} of ^{32}P nick-translated DNA probe (1-3 × 10^{8} cpm/μg^{-1}). Filters were washed twice for 30 min at 68 °C with the following solutions: 4 × SSC, 0.1% SDS, 1 × Denhardt's solution, 0.1% sodium pyrophosphate; 2 × SSC, 0.1% SDS; 0.2 × SSC, 0.1% SDS; 0.1 × SSC, 0.1% SDS. Autoradiography at −70 °C with a Cawo VI intensifying screen proceeded for about 3 days.

Results and discussion

Specificity of the detection of HLA-DR gene fragments. To achieve maximum discrimination between different *DR* haplotypes, we made use of a large genomic probe covering almost the entire DR_{β} chain gene (Fig. 1). To control for the specificity of DR_{β} hybridization, a Southern blot was made using DR_{β}, DQ_{β}, or DP_{β} genomic genes cloned in three different phages. The filter was hybridized with the nick-translated plasmid pP14 BamG (Fig. 2). Under the experimental conditions used, the probe hybridizes only with the cloned DR_{β} gene, and no cross-hybridization is visible with *DQ* or *DP* β genes. Overloading the gel with the same DR, DQ, and DP clones resulted in a slight cross-hybridization with the DP_{β} gene (Fig. 2). Densitometric analysis indicates that the DP_{β} signal is about 100-fold weaker than the DR_{β} signal. Under the conditions used to analyze genomic DNA, the DR_{β} probe hybridizes only to *DR* genes. In other studies,

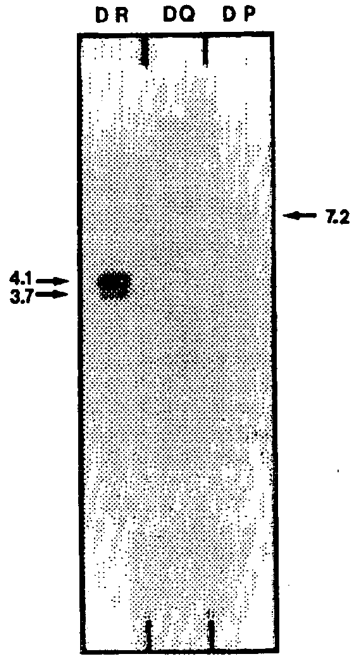


Fig. 2. Specificity of hybridization with the DR_{β} chain probe pP14BamG. A Southern blot was made using cloned genes coding for DR_{β}, DQ_{β}, and DP_{β} chain genes digested with Eco RI or Hind III (see *Materials and methods*). The filter was hybridized with the nick-translated DR_{β} chain plasmid pP14BamG (Fig. 1)

cross-hybridization of DR_{β} probes with *DQ* and DP_{β} chain genes has resulted in complex patterns which are often difficult to interpret at the molecular level (Cohen et al. 1984, Nicklas et al. 1985, Bell et al. 1985). It should be noted that the genomic probe pP14 BamG does not contain repetitive sequences and hybridizes only with DR_{β} gene fragments.

Definition and limits of allele-specific DNA patterns. DNA from genetically homozygous cell lines (Table 1) was used as references to define allele-specific DNA patterns. The results obtained with Bgl I have already been published (Angelini et al. 1984), and Figure 3 represents a summary of the reference patterns obtained with the enzymes Bgl I, Eco RI, and Pvu II. Homologous restriction fragment patterns are presented side by side (Fig. 3). It is striking that groups of very similar DNA patterns can easily be recognized with each of the three enzymes used: DR 3, 5, w6, w12, and DR4,7 respectively (Fig. 3). These two groups correspond to the well-known DRw52 and DRw53 supertypic groups characterized serologically (Tanigaki and Tosi 1982, Angelini et al. 1984). The haplotypes which could be classified in one of these two groups are therefore structurally related and they probably have a common origin in evolution (Gorski et al. 1986, 1987a). This observation may have some practical implications for an "intelligent" HLA-DR matching in organ transplanta-

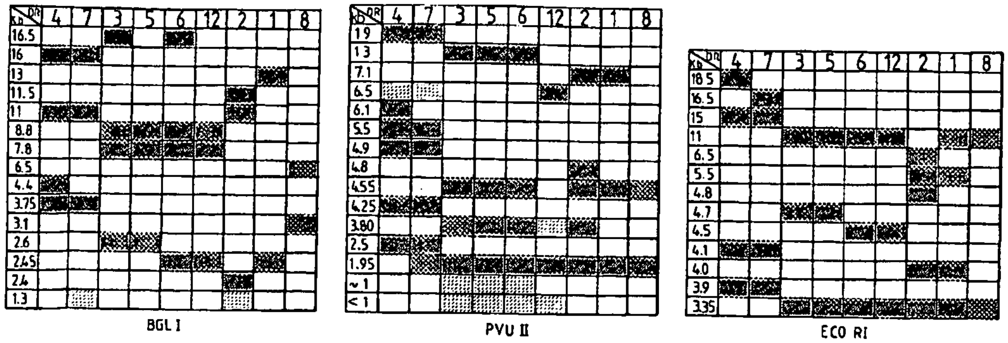
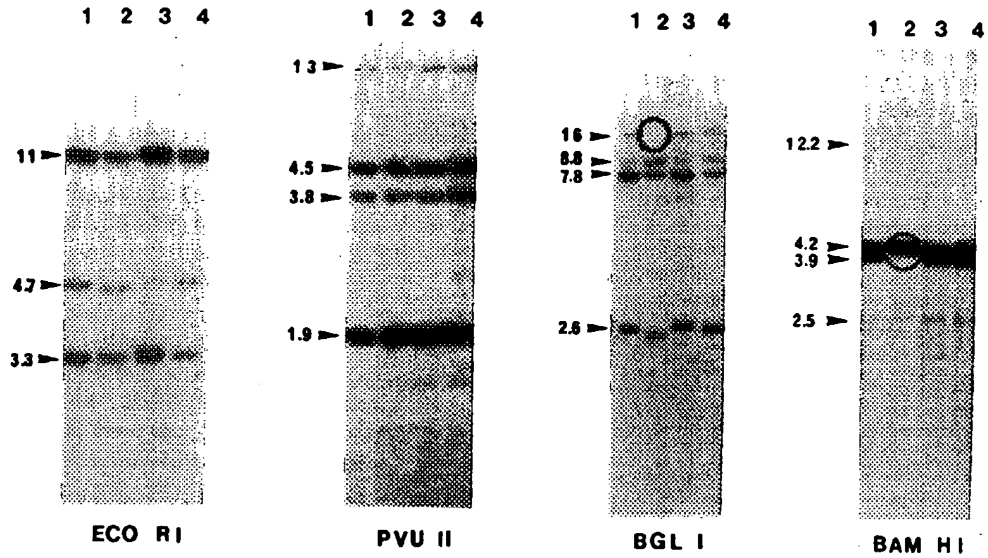


Fig. 3. Diagrammatic patterns of DR_β DNA fragment length of the homozygous typing cell lines (HTC) used as references. The origin and the typing of the DNA samples are summarized in Table 1 (AVL is used as DR 3/3 HTC). From left to right the DNA patterns were obtained with Bgl I, Pvu II, and Eco RI. These diagrams are based on Southern blot experiments as presented by Angelini and co-workers (1984). The *dark strips* represent major bands and the *dashed strips* represent fainter bands. The scale is in kilobases. A close similarity in two groups of DNA patterns is visible: DR 4/4, and DR 7/7 on one side (DRw53); DR3/3, DR5/5, DRw6/w6, and DRw12/w12 on the other side (DRw52)



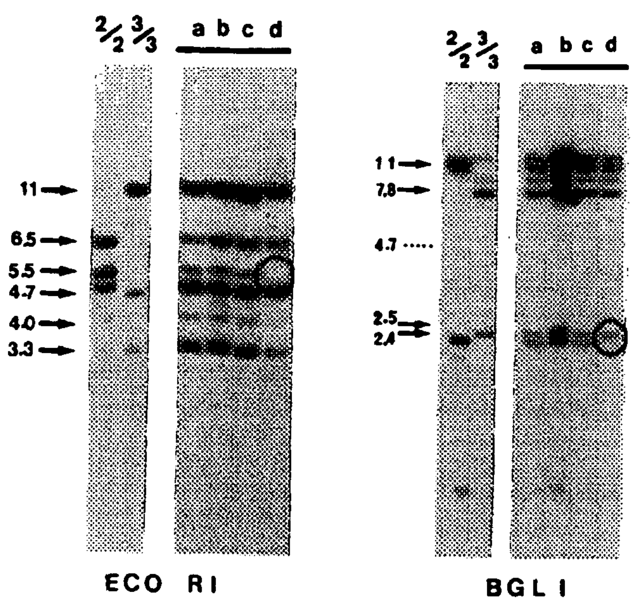


Fig. 4. Examples of genomic heterogeneity within the same serological specificities. *Upper panel:* DNA in lanes 1, 2, 3, and 4 were prepared from DR3/3 genetically homozygous cell lines AVL, QBL, HAR, and CAA (Table 1). DNA samples were digested with Eco RI, Pvu II, Bgl I, and Bam HI. The *circles* indicate the position of DNA bands that are missing in sample 2 (QBL). *Lower panel:* Comparison of DR_β chain gene restriction fragments from *DR2/3* heterozygous individuals. Samples a, b, c, and d correspond to the cell lines H9, M14, PM1, and R5 (Table 1). The *circles* indicate the position of DR2-specific DNA bands that are missing in sample d. Reference homozygous patterns are included for comparison

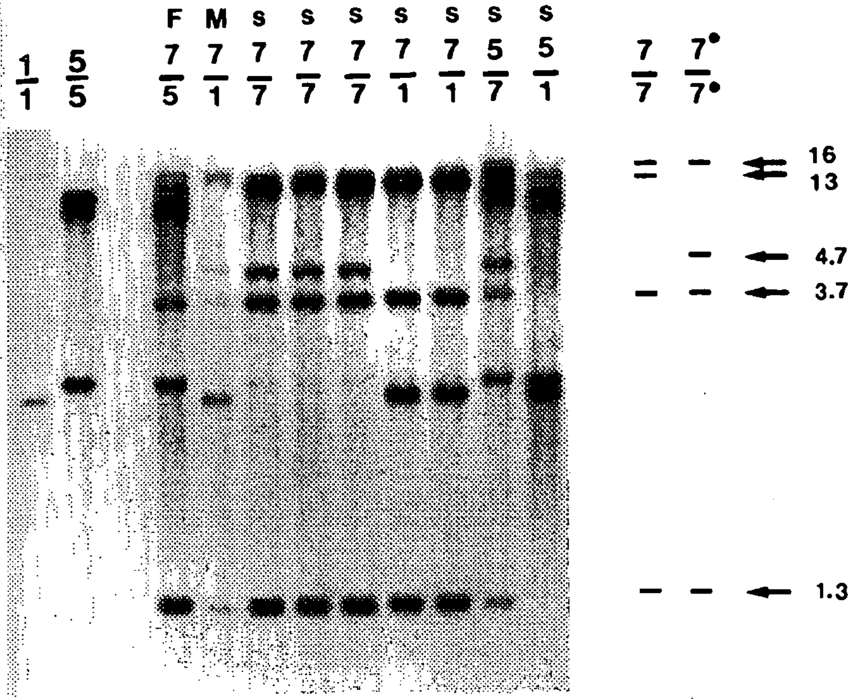
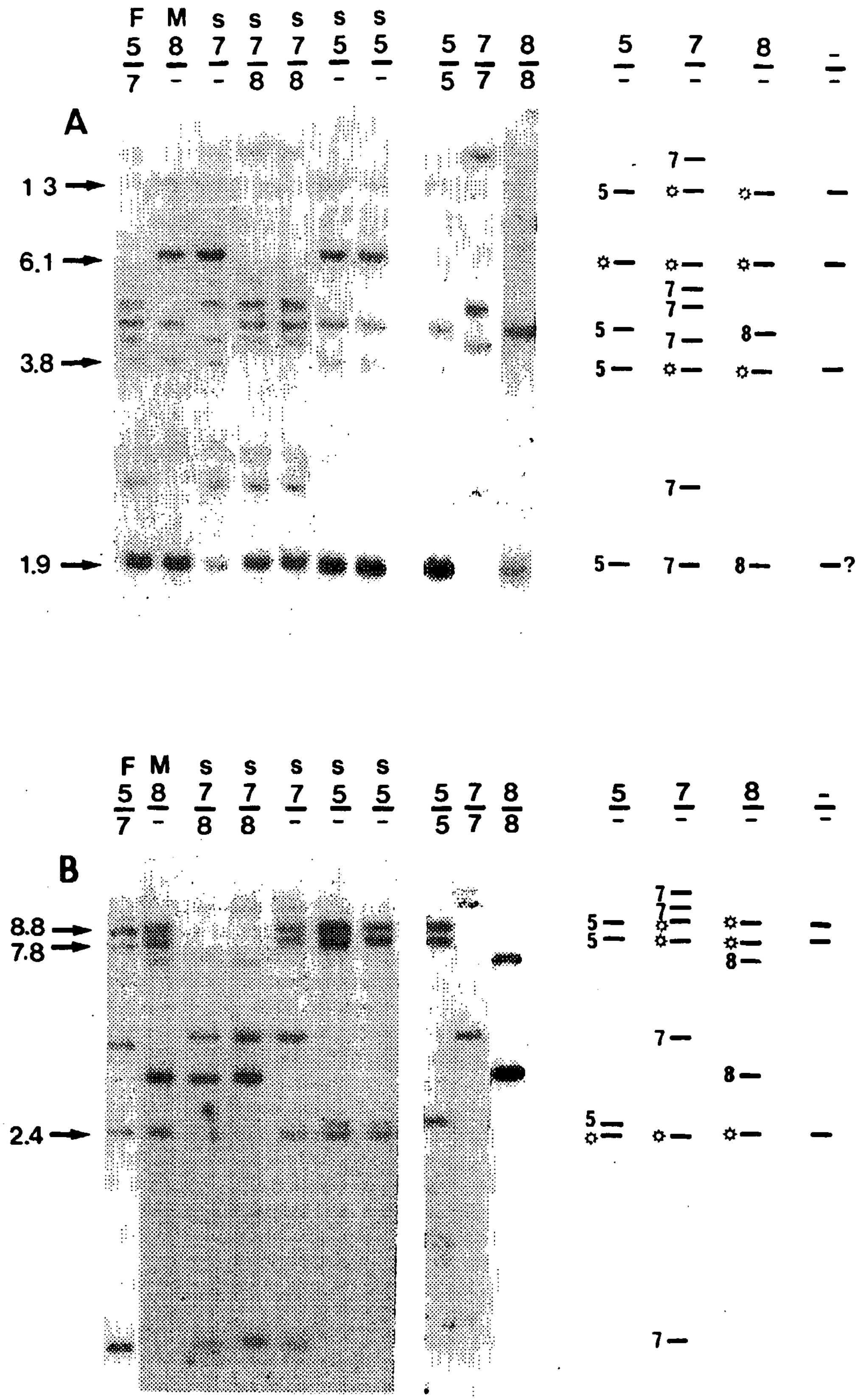


Fig. 5. Genomic heterogeneity of DR_β chain genes coding for the DR7 specificity. Haplotypes of family GR are: Father: *A25, Bw51, Cw1, DR5, DRw52, DQw3. A29, Bw44, DR7, DRw53, DQw2.* Mother: *A2, Bw51, DR7, -, DQw3. A24, Bw44, Cw2, DR1, DQw1.* DNA of the family members and of HTC DR1/1 and DR5/5 was digested with Bgl I. The DNA pattern transmitted by the *DR7* haplotype of the father corresponds to the pattern of the HTC MANN (Fig. 3) and is represented as 7/7. The DNA pattern transmitted by the DR7 haplotype of the mother is different and is represented on the scheme as 7•/7•

tion. Indeed we have proposed that a single HLA-DR mismatch across different supertypic groups will have greater consequences than a single mismatch within a given supertypic family, where the *DR* genes are structurally very similar (Mach et al. 1986).

In a few cases, we observed DNA patterns that differed slightly from the reference RFLP shown in Figure 3. Thus some serological specificities correspond to distinct alleles at the genomic level. The existence of such "splits" of serological specificities is well documented at the cellular level (Grosse-Wilde et al. 1984), at the biochemical level (Nepom et al. 1983), and at the DNA level (Se et al. 1985). Examples concerning *DR2* and *DR3* haplotypes are presented in Figure 4, and an example concerning *DR7* will be discussed in the next paragraph. DNA samples of four individuals known to be DR3 homozygous (consanguineous marriages) were analyzed with four restriction enzymes (Fig. 4, upper panel). These four samples share the same distribution of Eco RI and Pvu II restriction sites. However, with Bgl I and Bam HI, sample 2 (QBL) differs from samples 1, 3, and 4 (AVL, HAR, CAA, Table 1). A study of the restriction map of the cloned DR_β chain genes of AVL (sample 1) shows that the 3.9 kb Bam HI fragment which is missing in QBL (sample 2) corresponds to the $DR_\beta III$ locus (Rollini et al. 1985). Indeed, the haplotypes of AVL and QBL have since been found to differ at the $DR_\beta III$ locus by DNA sequence analysis (J. Gorski et al., manuscript in preparation) and by oligonucleotide hybridization (Angelini et al. 1986a, Gorski et al. 1987b) corresponding respectively to the *52a* and the *52b* allele at that locus (Gorski and Mach 1986). Another case of split of serological specificity with RFLP was observed on a DR2 pattern. Four DR2/3 DNA samples provided by S. Shaw were analyzed with Eco RI and Bgl I (Fig. 4, lower panel). In samples a, b, and c, DNA fragments correspond exactly to the sum of DR2/2 and DR3/3 reference patterns, whereas for sample d (cell line R5, Table 1), one DR2-specific fragment is missing on both Eco RI and Bgl I patterns. This difference may be correlated with a functional difference. Although cell line R5 is indistinguishable by serology from the other DR2/3 cell lines H9, M14, and PM1 (Table 1) (Shaw et al. 1980), it produces a strong mixed lymphocyte reaction when tested with cells from the other DR2/3 individuals (S. Shaw, personal communication). The split in *DR2* genotypes observed by RFLP (Fig. 4) correlates therefore with the functional differences observed in cellular reactivity. Additional polymorphism detected by RFLP is not necessarily relevant, however, since many polymorphic restriction sites are located in noncoding regions (introns and flanking regions). As discussed elsewhere (Mach et al. 1986), the analysis of polymorphic splits by hybridization with allele-specific oligonucleotides (Angelini et al. 1986a) does provide a way to identify phenotypically relevant micropolymorphism, down to the level of single nucleotide differences.

Discrimination between split of serological specificity, unknown DR allele, and homozygous DR genes. In many instances, only a single DR specificity is identified pheno-


A
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5/7 8/- 7/- 7/8 7/8 5/- 5/-
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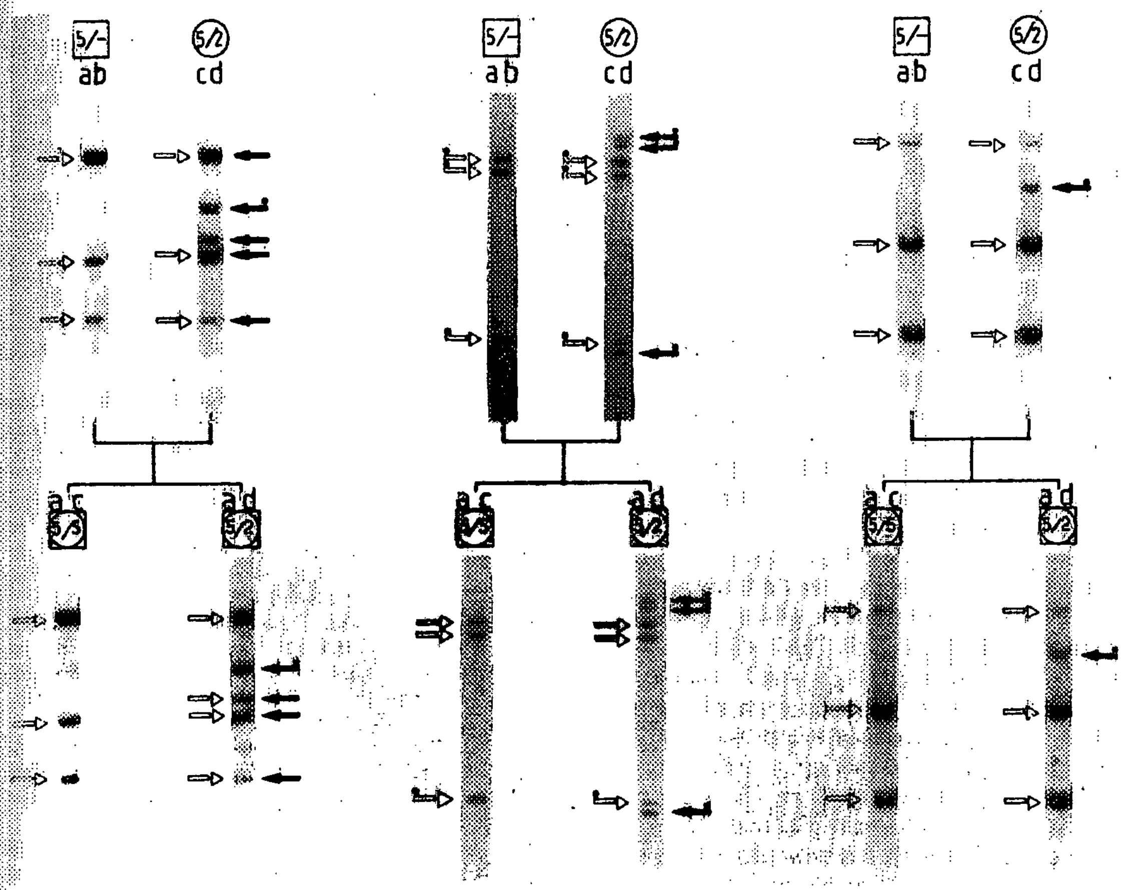

Fig. 7. Analysis at the DNA level of a *DR*-blank serological haplotype: example of a homozygous DR specificity. The serological haplotypes of family RG are: Father: *a*: *A2, Bw44, Cw5, Bw4, DR5, DRw52*. *b*: ? Mother: *c*: *Aw24, B7, Bw6, DR2, DQw1*. *d*: *A2, Bw56, Cw1, Bw6, DR5, DRw52, DQw3*. The serological typings indicate that three siblings, male and female, share the *a/c* haplotypes and two siblings, male and female, the *a/d* haplotypes. A single example of restriction patterns obtained with DNA from *a/c* or *a/d* offsprings is shown because no variation was found between haploidentical siblings. From left to right the DNA patterns were obtained with Eco RI, Bgl I, and Pst I. *Open arrows* correspond to DNA fragments from the *DR5* haplotype and *closed arrows* to DNA fragments from the *DR2* haplotype. The *dot* indicates that a given band can be used as a haplotype-specific marker in the context of the restriction fragments observed

typically upon HLA typing. It is essential to determine if those individuals are genotypically homozygous and if not, to identify the serologically undetected allele. We show here that this can be achieved by RFLP in family studies. Three different examples will be presented. (a) In family GR, the father and the mother were both typed *DR7* for one of their haplotypes (see legend to Fig. 5). Three siblings inherited both *DR7* alleles and were expected to be homozygous. However, as shown with Bgl I restriction fragments (Fig. 5), the parents differ in the structure of their $DR7_{\beta}$ genes. The DR pattern of the father is identical with the *DR7* of the cell line MANN, but the DR7 pattern of the mother lacks an 11 kb band and has an extra 4.7 kb fragment. In consequence, the *DR7* haplotypes of the father and the mother are different at the DNA level (see 7/7 versus 7'/7' in Fig. 5) and the DR7/7 sib-

◀ **Fig. 6A and B.** Analysis at the DNA level of *DR*-blank serological haplotype: Example of an undetected DR specificity. The serological haplotypes of family RU are: Father: *A28, B47, DR5, DRw52, DQw3. A29, B44, DR7, DQw2*. Mother: *A2, B18, DQw3. A2, B5, Bw4, DRw8, DRw52j*. The DNA patterns of family members and of three HTC used as references are presented in two panels: in A DNA digestion was performed with Pvu II and in B digestion was performed with Bgl I. The DR serological typing is indicated on the top of each lane (F, father; M, mother; s, siblings). On the right part of the figure the DNA patterns of all individuals with a DR-blank phenotype (5/-, 7/-, 8/-) are represented schematically. The *numbers* indicate the bands specific for DR 5, 7, or 8. The *starts* represent the additional DNA bands corresponding to the β chain genes of the *DR* blank haplotype. At the extreme right of the scheme the deduced DR DNA pattern of the blank haplotype (-/-) is represented both for Pvu II and Bgl I; the size of these genomic fragments is indicated on the left of the figure. On A it is not possible to know if the 1.9 kb fragment shared by *DR5, DR7*, and *DR8* haplotypes is also present on the *DR* blank haplotype (?)

lings are therefore heterozygous (7/7'). The genomic pattern of the *DR7'* allele described here in the mother is also different from an additional *DR7* allele recently observed by RFLP by Angelini and co-workers (1986b) and therefore corresponds to a third *DR7* allele, characterized by a unique Bgl I pattern. Interestingly, the *DR7* haplotype of the mother (*DR7'*) does not encode the supertypic specificity generally associated with DR7, DRw53. In this haplotype, it is likely that the product of the locus encoding DRw53 is not expressed, as suggested by the protein analysis of Karr (1986) in another DR7, DRw53$^-$ situation. A deletion of the locus encoding DRw53 has been suggested by the RFLP pattern in a similar case (Böhme et al. 1985).

(b) In family RU, serological analysis detected only a single *DR* allele in the mother and three siblings (see legend to Fig. 6). In these individuals, a DNA restriction fragment pattern corresponding to a novel, serologically "blank" haplotype could be identified. With the enzymes Pvu II and Bgl I, the DNA pattern of the *DR* blank haplotype was deduced from a comparison with the DNA of the control reference HTCs (Fig. 6). This comparison involved *DR5/5* with 5/- (sibling), *DR7/7* with 7/- (sibling), and *DR8/8* with 8/- (mother). This newly defined pattern of a *DR* blank haplotype consists of Pvu II bands at 13, 6.1, and 3.8 kb, and of Bgl I bands at 8.8, 7.8, and 2.4 kb. Interestingly, some of these bands are also found in the RFLP pattern of haplotypes belonging to the DRw52 supertypic group, even though this new allelic form of the *DR* subregion does not code for the DRw52 specificity. It should be noted that the DNA patterns of this novel blank specificity differ from the RFLP patterns described for another DR blank specificity in the family BON (Coppin et al. 1987). Analysis of family RU at the cellular and biochemical level is in progress to identify the molecules encoded by the *DR* blank genes.

(c) In family RG, the mother was typed DR5/DR2, whereas the father and three siblings were typed DR5 only. This combination allowed us to compare the restriction patterns of the DR5/5 offsprings (haplotypes *a/c* in Fig. 7) with the pattern of the father 5/- (haplotype *a/b* in Fig. 7). With the use of three enzymes, Eco RI, Bgl I, and Pst I, no difference between the banding pattern of the two DR5/5 offspring and the banding pattern of the father was observed. These results indicate that the father does not carry any additional blank haplotype and is indeed DR5/5 homozygous. In this example the reference to homozygous standards was not necessary.

The three examples reported for families GR, RU, and RG illustrate how the use of an analysis of DR_β chain genes by RFLP can allow a genetic interpretation of serological phenotypes in cases where HLA-DR typing detects only a single allele.

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